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Report

Regulation of Cytokinesis by the Formin cdc12p

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Summary

For successful cell division, cytokinesis must be properly timed to occur only after the segregation of chromosomes during mitosis. In the fission yeast *Schizosaccharomyces pombe*, contractile ring assembly initiates at the onset of mitosis, and ring contraction occurs concomitant with septation at the end of anaphase. Although many of the conserved factors necessary for ring assembly and regulation of cytokinesis have been characterized [1, 2], still little is known about cell-cycle regulation of events that initiate cytokinesis. The formin cdc12p is an essential ring component with a well-characterized function in F-actin assembly [3–6]. Here we show that overexpression of a cdc12p fragment bypasses normal cell-cycle controls and induces contractile ring assembly and sometimes even ring contraction and septation, all during interphase. Activation of cytokinesis occurs without the apparent activation of cell-cycle regulators such as polo kinase or the septation initiation network [7]. For this effect, cdc12p contributes at least two separable activities: actin assembly and one or more additional functions in cytokinesis initiation. These observations suggest that the formin cdc12p participates downstream of cell-cycle regulators in a network that drives the initiation of cytokinesis.

Results and Discussion

A Truncated cdc12p Protein Induces Ring Assembly and Cytokinesis during Interphase

The formin cdc12p is a contractile ring protein required for the assembly of actin filaments specifically for the ring [3–6]. Like other formins, the FH2 domain of cdc12p nucleates actin filaments and remains associated with the elongating barbed end, whereas the FH1 domain binds profilin-actin and delivers actin to the growing barbed end [8]. Other regions of the protein contribute to cdc12p targeting and function [5]. Unlike many other formins, there is little evidence that cdc12p is regulated by Rho-type GTPases or that its regulation requires an inhibitory DAD-like domain [5].

During a cdc12p structure-function study, we fortuitously discovered that expression of a truncated cdc12p allele induced inappropriate ring formation in interphase *S. pombe* cells. This construct encodes a cdc12 protein with a C-terminal truncation that removes sequences distal to the FH2 and the DAD-like domains, replacing them with GFP (Figure 1A). This mutant cdc12p was incapable of supporting colony growth as the sole cdc12 protein. When this protein was overexpressed in a wild-type background from an inducible promoter, nearly all cells displayed rings, as assayed by cdc12ΔC-GFP fluorescence (Figure 1B; see also Figure S1A available online). Because most cells in an asynchronous population are in interphase, we suspected that these rings were present in interphase cells. Using microtubules as a marker for cell-cycle stage, we found that almost all interphase cells exhibited cortical rings containing cdc12ΔC-GFP (Figures 1C and 1D; 90.2%, n = 500, Figure 1E). Rings were not seen in cells overexpressing full-length cdc12-GFP or another formin, for3-GFP (Figure 1E); thus, the phenotype does not result simply from the overabundance of formin protein. These interphase rings contained F-actin and, in some cells, were associated with a septum (Figures 1F and 1G); actin effects were specific to ring assembly, because there were no obvious changes in other F-actin structures.

Time-lapse imaging showed that rings formed de novo while cells were in interphase (Figure 1H). As in normal ring assembly, cdc12ΔC-GFP was often first present in one or few cytoplasmic spots that then spread to form rings (Figure 1H) [5]. Surprisingly, in about a third of these cases (34.1%, n = 264), cells proceeded to undergo ring contraction and septation, all during the same interphase (Figure 1I; Movie S1). In these cells, nuclear division did not occur, and thus the nucleus was cut or moved to one side upon division to form an anucleate cell compartment (Figures 1I and 1J; Figure S1A). In other cells, rings formed during interphase and were maintained until mitosis, at which time ring contraction and septation occurred with normal timing after anaphase. In addition, multiple rounds of ring assembly and contraction were sometimes observed within a single cell cycle (Figure 1J).

The interphase rings induced by cdc12ΔC had many of the molecular hallmarks of bona fide contractile rings. In addition to the cdc12ΔC protein, rings contained ring proteins such as F-actin (Figure 1F), rlc1p (myosin regulatory light chain, a marker for myosin II; Figure 2A), mid1p (anillin-related protein; Figure S2E), and ain1p (alpha-actinin; Figure S2A) [1]. Contracting rings were associated with septum deposition (Figure 1G) and accumulation of bgs4p (β-glucan synthase; Figure S2A). Formation of sterol-rich membranes at the division site also occurred in these interphase cells, as normally observed in dividing anaphase cells [9, 10] (Figure S2D). Thus, cdc12ΔC appeared to drive normal ring assembly and cell cleavage during interphase.

Cdc12p Acts in a mid1-Dependent Pathway

It has been recently proposed that there are two pathways contributing to ring formation in *S. pombe* [7, 11, 12]. The first pathway, which depends on nodes organized by mid1p, is thought to be the predominant mechanism in early mitosis [13, 14]. The second, which is driven by the SIN pathway in a manner independent of mid1p and nodes, operates predominantly in late mitosis and cytokinesis phases to maintain the ring and drive septation and ring contraction [7, 11, 12]. We found that interphase rings induced by cdc12ΔC formed through the first of these pathways, the mechanism normally operating in early mitosis. First, we observed that interphase rings formed from "nodes" [13, 15]. At early time points in the induction of cdc12ΔC, when rings were just beginning to form, myosin was seen in node-like structures (or loose immature

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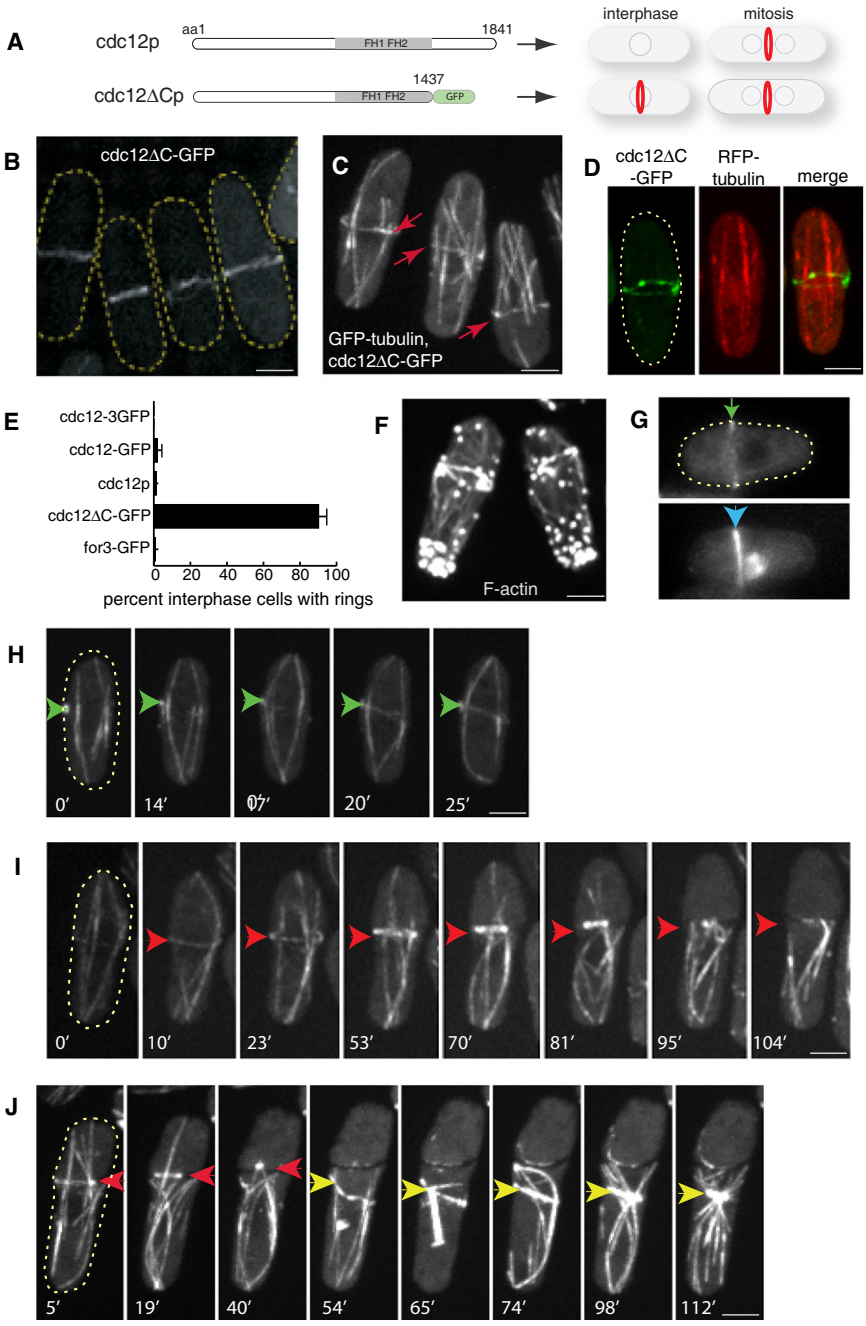

Figure 1. Expression of a Truncated Formin cdc12ΔC Induces Contractile Ring Formation in Interphase Cells

(A) Protein map and schematic localization of cdc12p and cdc12ΔCp.

(B) Cells (AY101) were induced for cdc12ΔC-GFP expression by growing them for 18–23 hr in thiamine-free media at 28°C. Maximum-intensity projections of confocal images (14 × 0.4 μm Z planes) are shown.

(C and D) Rings are present in interphase cells, as shown by the interphase microtubule distribution labeled by GFP- or mRFP-atb2 (AY120, AY517).

(E) Quantitation of rings in interphase cells expressing different formin constructs. Effect of formin constructs on the percentage (± standard deviation) of interphase cells expressing rings.

(F) F-actin in rings (arrows) as shown by Alexa-488 phalloidin staining.

(G) Wide-field image of a cell with an interphase cdc12ΔC-GFP ring (green arrow) associated with a septum stained with calcofluor (blue arrow) and an undivided nucleus stained with Hoechst (AY120).

(H–J) Time-lapse confocal images showing examples of cdc12ΔCp ring behavior and microtubule distributions; both structures are labeled with GFP (AY120).

(H) Ring formation from a spot in an interphase cell (arrow).

(I) Ring contraction (arrow) during interphase.

(J) Septation (red arrows) and ring contraction in interphase, followed by assembly and contraction of a second ring in mitosis and postmitosis (yellow arrows). Scale bars represent 2 μm.

rings) in nearly all interphase cells (94.5%, n = 200, <18 hr after thiamine removal; Figure 2B). In cells treated with latrunculin A (Lat A) to depolymerize F-actin, both rlc1p and cdc12p accumulated in node-like structures (Figure 2C); cdc12ΔC-GFP was detected in 40% ± 7% of rlc1-tomato-containing nodes in Lat A-treated cells (n = 10 cells). Second, ring formation was dependent on mid1p (Figure 2D) [16] and occurred around the nucleus (Figure S1D). Most interphase *mid1Δ* cells expressing cdc12ΔC formed disorganized, discontinuous filaments or abnormally positioned rings (89%).

Cdc12p Has Additional Functions Besides Actin Assembly

Next, we examined how cdc12 triggers ring formation. Is it primarily functioning to assemble actin filaments, or does it have additional ring-organizing activities? Increased actin assembly alone is not sufficient to induce ectopic ring formation. Overexpression of just the cdc12 FH1 and FH2 domains induces cytoplasmic assembly of F-actin but no actin rings [5, 6]. Similarly, other mutants that cause a general increase in actin assembly do not produce rings in interphase cells [17]. The ability of cdc12ΔC to assemble actin filaments was, however, required. We introduced mutations in the FH1 and FH2 domains of cdc12ΔC that inhibit actin assembly activity: a point mutation in the conserved actin-binding region of the FH2 domain (I1063A cdc12ΔC) [18, 19] and small deletions within the FH1 domain profilin-binding sites (cdc12ΔCΔPBD) [5] (Figure 3A). Expression of these mutant proteins in a wild-type background did not induce interphase ring formation (Figures 3A and 3B), suggesting that the actin assembly functions of the FH1 and FH2 domains are required. Moderate overexpression of cdc12-I1063AΔC did not otherwise interfere with cellular F-actin organization or actin ring assembly in mitotic cells (Figure 3D). However, importantly, these mutant proteins did cause myosin II and cdc12p to accumulate in node-like structures at the medial cortex (Figures 3B and 3C). The formation of these node-like structures was dependent on mid1p (Figure 3E), and thus were similar to node-like structures seen in Lat A-treated cells. Thus, cdc12p can activate aspects

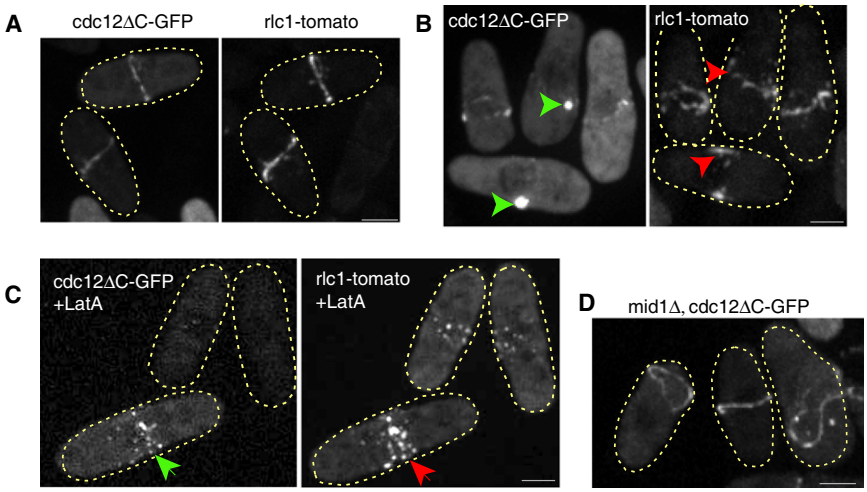

Figure 2. cdc12ΔC Forms Rings through a Normal Node-Dependent Mechanism

(A–D) Confocal maximum-intensity projection images are shown.

(A) rlc1-tomato localizes to interphase rings induced by cdc12ΔC-GFP (AY325).

(B) cdc12ΔC-GFP induces rlc1-tomato to form node-like structures (red arrows) prior to mature ring formation. Note that cdc12ΔC-GFP is primarily localized in spot structures in these cells (green arrows).

(C) Cdc12ΔC-GFP and rlc1-tomato colocalize in node-like structures (arrows) upon treatment with 100 μM latrunculin A (Lat A).

(D) Proper placement and assembly of the interphase cdc12ΔC-GFP rings are dependent on *mid1* (AY366). Scale bars represent 2 μm.

of ring formation, such as assembly of myosin II, into nodes [13, 16] independently of its ability to nucleate actin filaments.

To define this additional function of cdc12p, we tested different domains of cdc12p. First, we examined the function of the conserved FH1 and FH2 domains; do they primarily function in actin assembly, or might they have an additional function in triggering cytokinesis? We swapped the FH1 and FH2 domains of cdc12p with those of another formin, for3p, which is responsible for actin cable assembly in *S. pombe* [20]. The N-terminal half of cdc12p was fused with the C-terminal FH1 and FH2 domains of for3p (Figure 3A). Expression of this cdc12-for3 chimera induced formation of medial ring-like structures in interphase cells (50%, n = 100; Figures 3A and 3F; Movie S2). Like the rings induced by cdc12ΔC, these appeared to be bona fide rings: they contained F-actin and rlc1p and formed node-like structures upon Lat A treatment (Figures 3F–3H), although they were generally less well defined than wild-type rings and did not induce cleavage during interphase (n = 51). Thus, the for3 FH1 and FH2 domains can largely substitute for the cdc12p FH1 and FH2 domains, suggesting that the primary function of the FH1 and FH2 domains in ring assembly is in actin assembly rather than in a ring-specific function.

These results suggest that the remaining N-terminal portion of cdc12p encodes additional functions driving ring formation. To further define the relevant region, we found that a construct lacking the N-terminal 50 aa of cdc12ΔC (cdc12ΔNΔC-GFP) did not induce efficient interphase ring formation (Figures 3A and 3I), whereas cdc12ΔC, when expressed at equivalent or lower levels, did (Figures S1B and S1E). This N-terminal region was previously found to contain a nonessential targeting signal that contributes to cdc12p localization to rings in mitosis and thus is likely to interact with other ring components [5]. However, overexpression of N-terminal fragments alone was not sufficient to activate myosin accumulation to nodes (unpublished data). These findings indicate that cdc12p contributes at least two functions to drive cytokinesis: the FH1 and FH2 domains mediate actin assembly, and an N-terminal region provides actin-independent activities to promote the recruitment and activation of other ring proteins.

Cdc12p Acts Downstream of Other Cell-Cycle Regulators

Finally, we considered whether cdc12ΔC triggers ring formation by acting directly on structural components of the ring or through inappropriate activation of a cell-cycle regulator. It is unlikely that cdc12ΔC causes premature activation of the cdc2-cyclin B, because other aspects of mitosis, such as spindle formation, did not accompany ring formation (Figure 1). It is known that the SIN pathway can drive ring formation: mutants that have a hyperactive SIN pathway (e.g., *cdc16* loss-of-function mutants) undergo repeated rounds of ring assembly and septation even during interphase in a cdc12-dependent manner [7, 11, 21–25].

We tested whether cdc12ΔC might cause hyperactivation of the SIN pathway. Because the ability of cdc12ΔC to induce rings was itself thermosensitive, we were unable to test for SIN function with existing temperature-sensitive *sin* mutants. Instead, we examined the activity of the SIN pathway by assaying the localization of marker proteins cdc7p, sid2p, mto1p, and myp2p [26–30]. In wild-type cells, the SIN is most active in mid and late mitosis through cytokinesis. In cells expressing cdc12ΔC, the behavior of markers indicated that although the SIN was active in mitosis, it was not activated in interphase (Figure 4; Figures S2B and S3). Factors such as sid2p, myp2p, and mto1p that require SIN activity for ring localization in midmitosis were not detectable on any of the cdc12ΔC interphase rings. Thus, cdc12ΔC does not cause ring formation by hyperactivating the SIN pathway. One interesting finding is that the SIN pathway did not appear to be active even in the subset of interphase cells that exhibited ring contraction and septation, suggesting that the SIN pathway activation was bypassed in this circumstance (Figure S3). In addition, cdc12ΔC activity was not dependent on the CDC14-like phosphatase clp1p/flp1 and did not cause exit of clp1p from the nucleolus (Figure 4; Figure S3), providing further evidence that the effects are not a consequence of an activated cytokinesis checkpoint response [31, 32]. Another conserved regulator of cytokinesis and mitosis is the polo-like kinase plo1, which is activated at the spindle pole body [33–36]. We observed no apparent recruitment of plo1p to spindle pole bodies of interphase cells with rings (Figure 4D), suggesting that plo1p is not activated by cdc12ΔC. One function of plo1p is to trigger the nuclear export of mid1p at the onset of mitosis [37]. In the cdc12ΔC expressing interphase cells, much of mid1p was still nuclear, and cells exhibited a mild defect in ring positioning, as seen with weak mutant alleles of plo1, providing further evidence that plo1p is not functionally highly active in these cells (Figures S1D and S2F). These findings suggest that

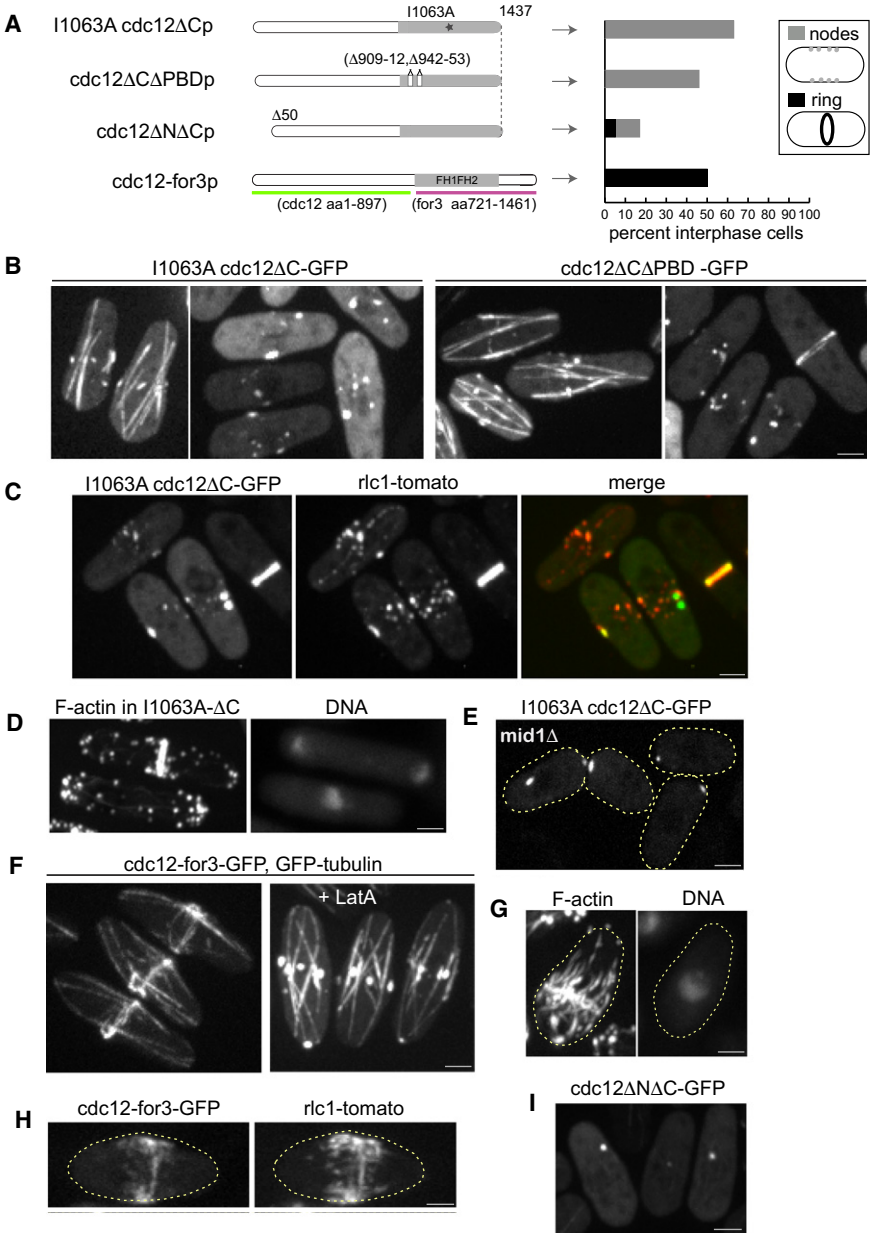

Figure 3. Cdc12p Contributes Multiple Activities for Cytokinesis

(A) Schematic of *cdc12* mutant constructs and quantitation of localization patterns; n = 100.

(B–F) Confocal maximum-intensity projection images.

(B) *cdc12* actin assembly mutants I1063A cdc12ΔC and cdc12ΔCΔPBD, with GFP-tubulin (left, each pair) or without (right, each pair), localize to medial cortical dots (arrows) in interphase.

(C) Colocalization of I1063A cdc12ΔC-GFP with rlc1p in node-like structures.

(D) Alexa phalloidin staining of cells expressing I1063A cdc12ΔC (AY390).

(E) Mutant cdc12p is not in node-like structures in a *mid1*Δ background (AY622).

(F) Expression of a chimeric fusion protein cdc12-for3-GFP produces rings (AY443) in interphase cells, as shown by GFP-tubulin (left). Cdc12-for3p localizes to node-like structures in these cells upon Lat A treatment (right).

(G) Phalloidin staining for F-actin in cells expressing the chimera cdc12-for3.

(H) Rlc1p-tomato localizes to cdc12-for3-GFP ring structures (AY508).

(I) Absence of rings in cells expressing cdc12ΔNΔC-GFP (pAY51). Scale bars represent 2 μm.

cdc12ΔC does not activate ring assembly by inappropriately activating these cell-cycle regulatory pathways.

It is likely that cdc12p participates in a regulatory network that collectively triggers the division process. Overexpression of cdc12ΔC could plausibly somehow activate a number of cytokinesis factors involved in ring initiation, such as mid1p and myosin type II complexes. One candidate protein in this initiation network is cdc15p, an F-BAR domain-containing protein that interacts with the N-terminal region of cdc12p [38, 39]. cdc15p is not strictly required for normal ring assembly but is thought to be required for more robust maintenance of rings, especially late in cytokinesis [12, 40]. Overexpression of cdc15p also induces accumulation of medial actin filaments in interphase, although less efficiently than cdc12ΔC and often in more aberrant aggregate-like structures rather than in discrete rings [25, 41] (unpublished data). Although we could not easily test a *cdc15* null allele because it is essential for viability, we found that cdc12ΔC expression did not induce ring formation in a strain expressing cdc15-tomato, a fusion protein that may be only partially functional [11] (3% of interphase cells exhibited cdc12-GFP rings, n = 200). This initial observation suggests that cdc15p is needed for ring formation in this circumstance.

In summary, we show here that expression of a truncated cdc12p protein drives cytokinetic events at inappropriate stages of the cell cycle. These findings provide a striking demonstration that cytokinesis can be uncoupled from other cell-cycle events, showing that cytokinesis is not necessarily dependent on mitosis. Although cdc12p has been widely assumed to be primarily an actin assembly factor, our data demonstrate that cdc12p has broader roles in coordinating other events in cytokinesis (Figure 4F). These functions are consistent with the observation that in cdc12 null cells, both actin ring assembly and septum formation are completely absent, whereas loss of actin alone by Lat A treatment still leads to deposition of septal materials [3]. cdc12ΔC does not activate ring assembly by inappropriately activating these cell-cycle regulatory pathways, such as CDK1 or the SIN pathway, but may act downstream to trigger cytokinesis initiation by acting directly or indirectly on a network of ring factors including actin, cdc15p, myosin type II, and mid1p (Figure 4F). How the cell cycle regulates cdc12p, presumably through the C-terminal domain of cdc12p, is not yet known. Cell-cycle changes in cdc12 protein levels or mobility have not been detected (F.C., unpublished data). Further characterization of cdc12p formin regulation and its functional interactions will be fruitful for defining the networks at the interface

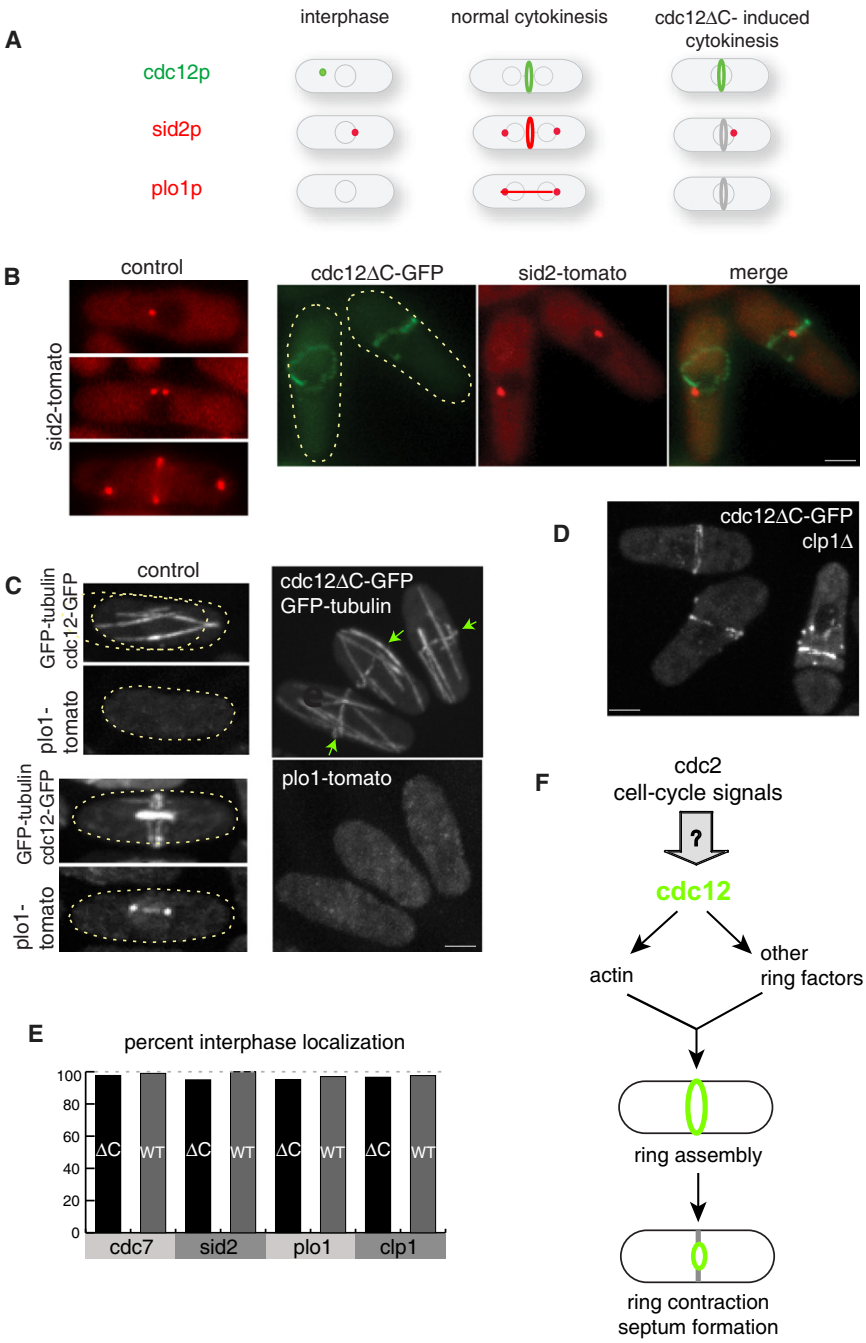

Figure 4. cdc12ΔC Expression Does Not Activate the SIN or Polo Kinase Cell-Cycle Regulators

(A) Schematic showing localization patterns of cdc12p, sid2p (SIN pathway marker), and plo1p polo kinase.

(B) Sid2-tomato in wild-type cells (left, AY250 in interphase [top], mitosis [middle], and cytokinesis [bottom]) and in cdc12ΔC-GFP interphase cells (right, AY235).

(C) Plo1p localization in wild-type cells expressing GFP-tubulin (left, AY462 in interphase [top] or mitosis [bottom]) and in cdc12ΔC-GFP-expressing cells in interphase (right).

(D) Interphase cdc12ΔC-GFP rings present in *clp1*Δ cells. Scale bars represent 2 μm.

(E) Quantitation of localization patterns showing percentage of interphase cdc12ΔC-GFP and wild-type cells that exhibit the interphase localization pattern of the indicated markers.

(F) Model in which cdc12p contributes to the initiation of cytokinesis by promoting actin assembly via its FH1 and FH2 domains and activation of other ring factors through the N-terminal region.

between the cell cycle and the structural proteins that carry out cell division.

Experimental Procedures

cdc12ΔC-GFP and related proteins were expressed in *S. pombe* from pREP42-derived plasmids from a thiamine-repressible medium-strength *nmt1** promoter. To induce expression, we grew cells at 28°C in Edinburgh minimal media (EMM) with appropriate auxotrophic supplements and thiamine to midlog phase (OD_{600} < 0.6) and then in EMM without thiamine for 18–22 hr. Cells were analyzed 20 hr or more after the shift to media without thiamine, except in Figures 2B and 2C, where cells were imaged at 18 hr. Details of strain and plasmid construction, other methods, and a list of strains are presented in the Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, one table, and one movie and can be found with this article online at doi:10.1016/j.cub.2010.01.061.

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